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Citation for published version:

Ortega, XP, Cardona, ST, Brown, AR, Loutet, SA, Flannagan, RS, Campopiano, DJ, Govan, JRW & Valvano, MA 2007, 'A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability', *Journal of Bacteriology*, vol. 189, no. 9, pp. 3639-3644.
<https://doi.org/10.1128/JB.00153-07>

Digital Object Identifier (DOI):

[10.1128/JB.00153-07](https://doi.org/10.1128/JB.00153-07)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Bacteriology

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A Putative Gene Cluster for Aminoarabinose Biosynthesis Is Essential for *Burkholderia cenocepacia* Viability[▽]

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Received 30 January 2007/Accepted 20 February 2007

Using a conditional mutagenesis strategy we demonstrate here that a gene cluster encoding putative aminoarabinose (Ara4N) biosynthesis enzymes is essential for the viability of *Burkholderia cenocepacia*. Loss of viability is associated with dramatic changes in bacterial cell morphology and ultrastructure, increased permeability to propidium iodide, and sensitivity to sodium dodecyl sulfate, suggesting a general cell envelope defect caused by the lack of Ara4N.

Cationic antimicrobial peptides (APs), a group of structurally diverse molecules found in all eukaryotes (17, 34), can kill gram-positive and gram-negative bacteria, fungi, and viruses and also modulate innate immune responses (30). In gram-negative bacteria, APs interact with lipopolysaccharide (LPS) and via the self-promoted uptake pathway reach targets in the plasma membrane (16). LPS is a major surface component of gram-negative bacteria, consisting of O antigen polysaccharide, core oligosaccharide, and lipid A (29). Phosphate groups covalently attached to residues on the lipid A and core oligosaccharide provide sites for electrostatic interactions with APs (18). Bacteria can resist APs by modifying their lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N), effectively reducing the net negative charge of LPS molecules. In all species examined to date, this is a highly regulated process in response to environmental Mg²⁺ limitation, excess Fe³⁺, or the presence of APs (1, 8, 9, 12, 15, 24). However, LPS modification with Ara4N is dispensable for growth in vitro under routine laboratory conditions.

Burkholderia cenocepacia belongs to the *B. cepacia* complex, a group of closely related environmental bacteria that are emerging opportunistic pathogens of cystic fibrosis patients and other immunocompromised individuals (23). They are highly resistant to the majority of clinically useful antimicrobials and also resistant to APs even at concentrations that kill other bacteria with Ara4N-modified LPS (21). Very little is known about the mechanism of resistance to APs in *Burkholderia* spp. LPS from various *Burkholderia* species, such as *B. cepacia*, *B. caryophylli*, and *B. cenocepacia*, contains Ara4N residues in the lipid A and also the unusual *glycero-talo*-octulosonic acid (Ko) residue in the inner-core oligosaccharide (10, 13, 19, 20, 25, 26, 31, 32). Here, we show that a putative gene

cluster for Ara4N biosynthesis and LPS modification is essential for the viability of *B. cenocepacia*.

A putative gene cluster for Ara4N biosynthesis in *B. cenocepacia*. A region spanning nucleotides 2128443 to 2135736 in chromosome 1 of *B. cenocepacia* strain J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) contains genes encoding proteins that are highly similar to those required for Ara4N biosynthesis and lipid A modification in *Salmonella* and other bacteria (14) (Fig. 1A). These genes correspond to homologues of *arnT* (*pmrK*, Ara4N transferase), BCAL1930 (*pmrLM*, unknown function), *arnB* (*pmrH*, UDP-4'-ketopentose aminotransferase), *arnC* (*pmrF*, Ara4N Und-P transferase), and BCAL1935 (*pmrJ*, unknown function) (Fig. 1A). Also, unlike other bacteria in which *arnA* (*pmrI*) is a single gene encoding a bifunctional enzyme (3, 4), two distinct genes are found in *B. cenocepacia* which we have designated *arnA1* (UDP-Ara4N formyltransferase) and *arnA2* (UDP-4'-keto-5'-carboxypentose decarboxylase) (Fig. 1A). The transcriptional organization of the putative *B. cenocepacia* *arn* gene cluster was experimentally determined by nested reverse transcriptase PCR (RT-PCR) amplifications, as previously described (27). We prepared cDNA from total RNA isolated from strain J2315 grown to logarithmic phase in Luria-Bertani broth with or without polymyxin B (100 µg/ml) and amplified it by PCR using primer pairs that would allow detection of cotranscription. All PCRs were performed alongside appropriate controls, including a non-RT control to assay for genomic DNA contamination. Primers used for RT-PCR analysis are available from the authors upon request. These experiments demonstrated that the putative *arn* genes are organized into two transcriptional units (Fig. 1B). One unit comprises *arnT* and BCAL1930, while the other includes the *arn* gene loci BCA1A2, BCAL1935, and BCAL1936 (Fig. 1A). This genetic organization suggests that the *B. cenocepacia* genes for the synthesis of UDP-Ara4N (*arn* BCA1A2) are transcribed independently from those for transferring Ara4N to the lipid A (*arnT* and possibly BCAL1930). The same genetic organization of the *arn* cluster was demonstrated for *B. cenocepacia* strain K56-2 (data not shown), which is clonally related to J2315 (22) but is more amenable to ge-

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[▽] Published ahead of print on 2 March 2007.

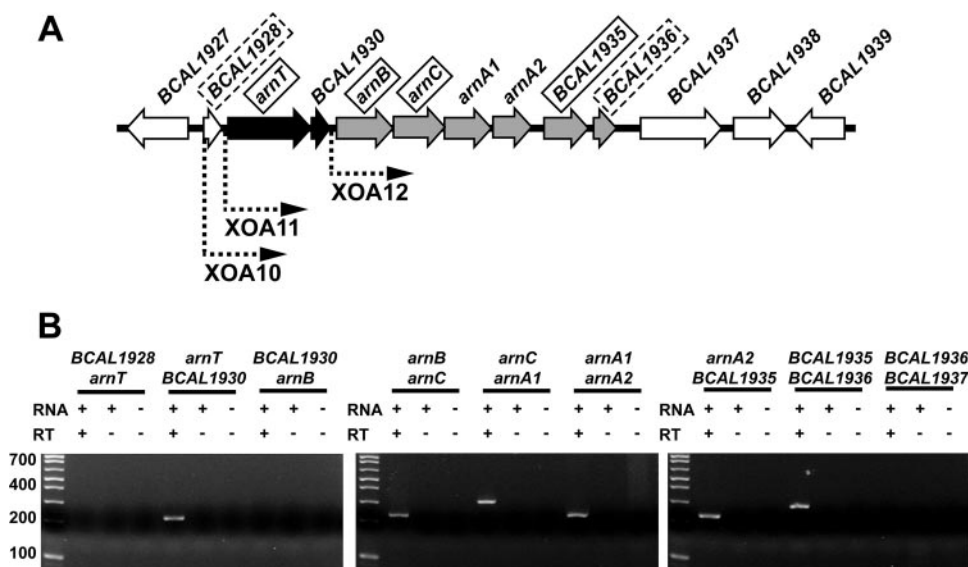


FIG. 1. (A) Genetic organization of the putative *arn* gene cluster and flanking regions on chromosome 1 of *B. cenocepacia* strains J2315 and K56-2. Solid boxes around gene and locus names indicate the genes for which polar mutations failed; dotted boxes indicate genes for which the creation of polar mutations was successful. Mutagenesis of the remaining genes was not attempted. The dotted arrows indicate the locations of the inserted rhamnose-inducible promoter in strains XOA10, XOA11, and XOA12. Black and gray arrows denote the genes within the two transcriptional units of the putative *arn* cluster that were identified by RT-PCR. (B) RT-PCR analysis of the *arn* cluster, performed on strain J2315 in the absence of polymyxin B challenge.

netic manipulations. RT-PCR analysis also revealed that the *arn* cluster in both strains was expressed regardless of polymyxin B challenge (data not shown), indicating that it is not subjected to the same transcriptional regulation as has been observed in all other bacteria examined to date (1, 24). To facilitate genetic manipulations the remaining studies were performed using strain K56-2, which is also highly resistant to APs (21).

The putative Ara4N biosynthesis cluster is essential for viability of *B. cenocepacia*. Repeated attempts to mutagenize *arnT*, *arnB*, *arnC*, and BCAL1935 by insertional inactivation using the pGpΩTp suicide plasmid (11) containing internal fragments from each target gene consistently failed. In contrast, the genes BCAL1928 and BCAL1936 that flank each end of the putative *arn* cluster were readily mutated. We hypothesized that the putative *arn* genes perform one or more functions that are essential for the viability of *B. cenocepacia*. To confirm the essentiality of the *arn* genes, we constructed plasmid pSC200, which enables the delivery of the rhamnose-inducible *P_{rhaB}* promoter into the chromosome to drive the expression of a targeted gene. The construction of pSC200 was done by combining the multiple-cloning site, *ori_{R6K}*, and *mob* genes from pGpΩTp (11) with the *P_{rhaB}* rhamnose-inducible promoter, *rhaR*, *rhaS*, and the *dhfr* cassette from pSCrhaB2 (7). Details on the construction of this plasmid are available from the authors upon request. Several 300-bp fragments spanning the 5' region of each targeted gene were cloned into pSC200. The resulting plasmids were transferred into *B. cenocepacia* K56-2 by triparental mating, and the exconjugants were plated on LB agar plates supplemented with trimethoprim (Tp) (100 µg/ml), gentamicin (50 µg/ml), and 0.5% (wt/vol) rhamnose. The correct insertion in the *B. cenocepacia* chromosome was confirmed by colony PCR and Southern blot hybrid-

ization. This strategy created conditional mutants in which the expression of the targeted gene depended on the rhamnose concentration in the medium. We have shown that the *P_{rhaB}* promoter is useful for characterizing essential operons in *B. cenocepacia* (6). Thus, we constructed conditional mutants by insertion of pSC200 derivatives upstream of *arnT* (strain XOA11) and *arnB* (strain XOA12) as well as a mutant in which this plasmid interrupts the gene locus BCAL1928 (strain XOA10) and which served as a negative control (Fig. 1A). Also, as a positive control for essentiality, a pSC200 derivative was used to target the conserved essential gene *dxs* encoding a key enzyme for the synthesis of undecaprenyl phosphate (2, 28), resulting in strain STC280.

The conditional mutants were grown at 37°C in M9 minimal medium (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 8 mM NaCl, 10 mM NH₄Cl) supplemented with (final concentrations) yeast extract (5 mg/ml), Casamino Acids (2 mg/ml), vitamin B₁ (2 µg/ml), tryptophan (20 µg/ml), CaCl₂ (1 µM), and 0.5% (wt/vol) glycerol plus 0.5% (wt/vol) glucose or 0.5% (wt/vol) rhamnose and Tp (100 µg/ml) when required. An aliquot of an overnight culture in M9 medium with rhamnose was spun down and washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an optical density at 600 nm (OD₆₀₀) of 1. Drops (10 µl) of undiluted solution and 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ dilutions were plated onto M9 agar plates supplemented with 0.5% (wt/vol) glucose or 0.5% (wt/vol) rhamnose and incubated at 37°C. Strain STC280 grew in the presence of rhamnose (permissive condition) but failed to grow in the presence of glucose (nonpermissive condition), as expected for a mutant with an essential gene under the control of *P_{rhaB}*. A similar growth phenotype was found for XOA11 and XOA12, but XOA10 grew equally well in the presence of rhamnose or glucose (Fig. 2A). We

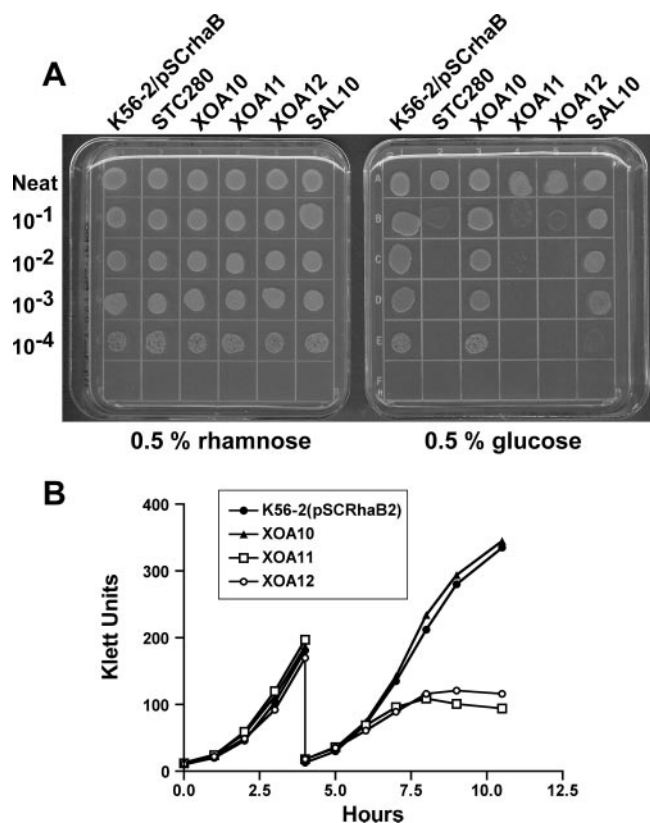


FIG. 2. (A) Conditional lethal phenotype of strains XOA11 and XOA12 on M9 agar plates supplemented with 0.5% (wt/vol) rhamnose or 0.5% (wt/vol) glucose. XOA11 and XOA12 grow only in the presence of rhamnose, while XOA10 and SAL10 grow equally well in either media. STC280 is shown as a control mutant for a known essential gene. (B) Depletion experiments using strains K56-2(pSCRhaB2), XOA10, XOA11, and XOA12 in nonpermissive conditions. Growth was monitored every hour using a Klett-Summerson colorimeter. The figure is representative of two independent experiments with similar results.

therefore concluded that expression of one or more genes in the two transcriptional units of the putative *arn* cluster is indispensable in *B. cenocepacia* K56-2.

The first biochemical reaction for the biosynthesis of Ara4N is the conversion of UDP-glucose into UDP-glucuronic acid by the enzyme UDP-glucose 6-dehydrogenase (Ugd or PmrE). In previous work, we characterized a gene operon containing a *ugd* homologue (BCAL2946) and genes encoding modification of *glycero-manno-heptose*, which are part of the biosynthesis of the ADP-*glycero-manno-heptose* precursor for core oligosaccharide biosynthesis (21). To assess whether or not *ugd*_{BCAL2946} is also essential, we constructed a conditional *ugd*_{BCAL2946} mutant (SAL10). This mutant did not show a conditional lethal phenotype in medium with glucose (Fig. 2A), indicating that *ugd*_{BCAL2946} is not essential for bacterial viability. A BLAST analysis of the *B. cenocepacia* J2315 genome revealed the presence of two additional *ugd* homologues in chromosome 2 (BCAM0855 and BCAM2034), which we assume provide redundant UDP-glucose dehydrogenase activities.

Loss of viability is associated with morphological changes in the bacterial cells and defects in cell envelope permeability.

The essentiality of Ara4N synthesis was further demonstrated by depletion experiments performed using liquid medium (Fig. 2B). Eight-hour cultures were diluted to an OD₆₀₀ of 0.001 in M9 medium supplemented with 5% (wt/vol) yeast extract and 0.5% (wt/vol) rhamnose and incubated at 37°C for 11 h. Cells from these cultures were spun down, washed three times with sterile PBS, and diluted with fresh M9 medium supplemented with 5% (wt/vol) yeast extract and 0.5% (wt/vol) glucose at a final OD₆₀₀ of 0.13. Growing cells were subcultured with fresh medium containing 0.5% (wt/vol) glucose at 4 h and monitored for growth for another 6 h. Growth curves of the wild-type strain and all the mutants in permissive conditions were identical (data not shown). After serial passages in medium with glucose, the wild type and the XOA10 mutant also grew to similar levels (Fig. 2B). In contrast, after serial passage in medium with glucose of XOA11 and XOA12 cultures, the turbidity of the cultures rapidly reached a plateau compared to wild-type and XOA10 control strain results. The results indicate that XOA11 and XOA12 had stopped growing after 6 h (roughly corresponding to six generations). Phase-contrast microscopy and fluorescent staining with Syto9 and propidium iodide (LIVE/DEAD *BacLight* bacterial viability kit; Molecular Probes, Invitrogen Detection Technologies, Eugene, OR) were carried out to investigate the bacterial cell morphology and to assess whether or not the cell envelope was compromised. After 8 h in nonpermissive conditions, strains XOA11 and XOA12, but not K56-2 and XOA10, formed chains of cells and the cultures contained flocculent particulate material indicative of cell lysis. Also, 50% of XOA11 and 58% of XOA12 bacterial cells were permeable to propidium iodide in contrast to 5% to 10% of the control strains, suggesting a loss of viability and a compromised cell envelope in the *arn* conditional mutants (Fig. 3). No differences in morphology or viability were observed between these same strains grown overnight under permissive conditions before being transferred to the nonpermissive medium for the depletion experiments (data not shown).

Ultrastructural analysis of bacterial cells after 8 h following depletion revealed accumulations of membranous material in strains XOA11 and XOA12 (Fig. 4), suggesting that Ara4N is required for the proper assembly of the outer membrane. Furthermore, empty cells and cells with cell division defects could be observed in the mutant cultures (Fig. 4). To obtain further evidence that the envelope was compromised in XOA11 and XOA12 under nonpermissive conditions, bacteria were grown in the presence of 0.05% (wt/vol) sodium dodecyl sulfate (SDS). For these experiments, cells were diluted to an OD₆₀₀ of 0.1 and dilutions were plated for colony counts on M9 agar plates supplemented with 5% (wt/vol) yeast extract, 0.5% (wt/vol) rhamnose, and Tp (100 µg/ml) with or without 0.05% (wt/vol) SDS. The survival rate of K56-2 cells in the presence of SDS was 1.2% of the initial inoculum, while the survival rates of XOA11 and XOA12 cells were 0.09% and 0.08%, respectively. Together, these experiments demonstrate that the putative *arn* gene cluster is essential for *B. cenocepacia* viability and that its function is required for an intact cell envelope. It is possible that LPS modifications with Ara4N are an essential requirement for the stability of the outer membrane in this

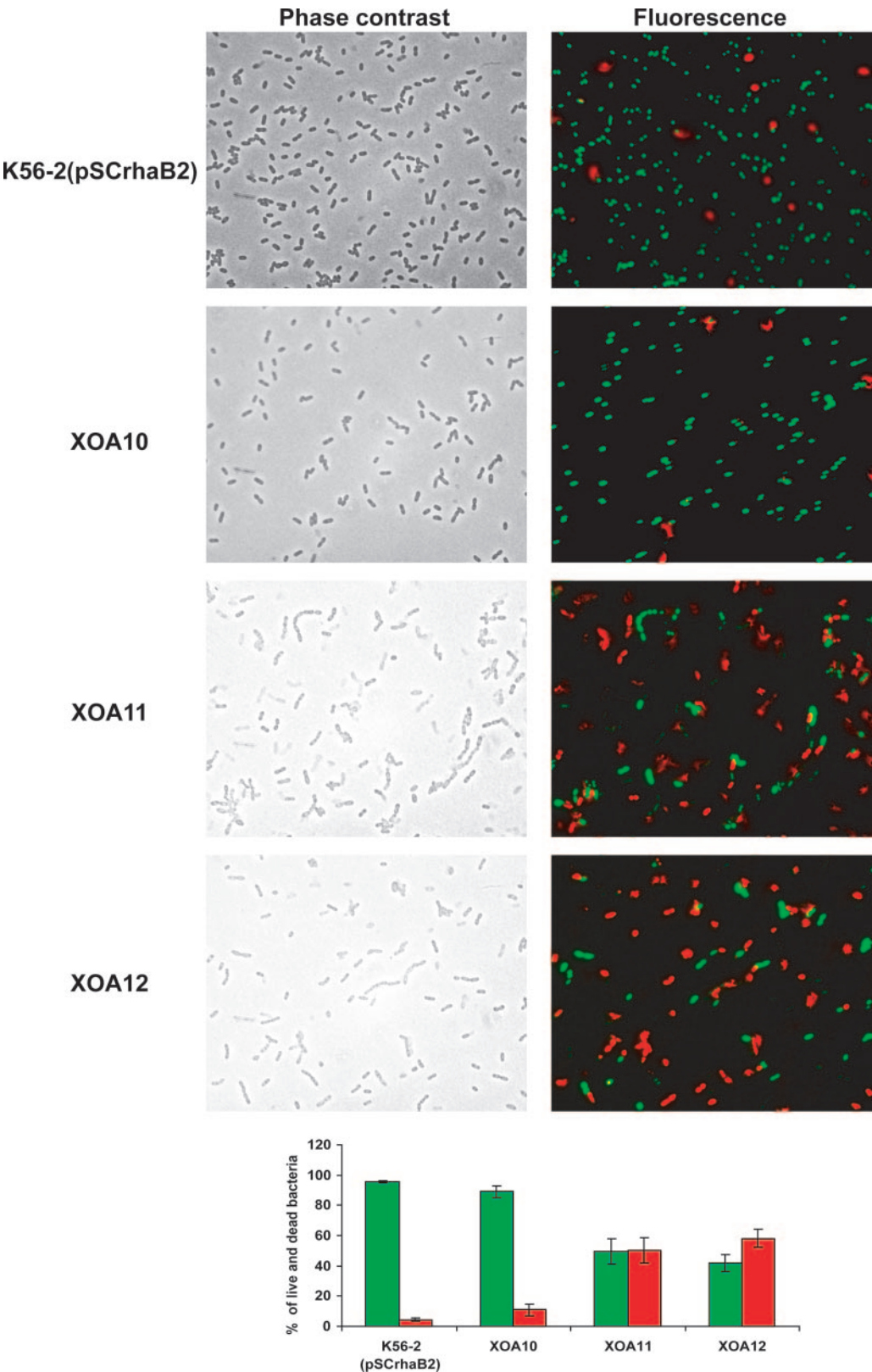


FIG. 3. Microscopy and live-dead staining of strains K56-2, XOA10, XOA11, and XOA12 at 8 h of growth during the depletion experiments. Live bacteria appear fluorescent green, while dead bacteria and bacteria with compromised membranes appear fluorescent red. These experiments were carried out twice with similar results.

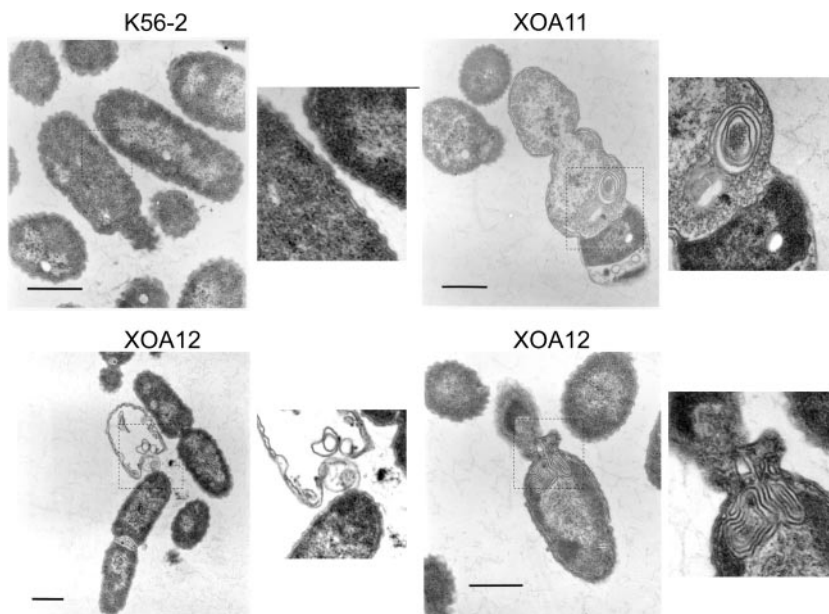


FIG. 4. Transmission electron microscopy of strains K56-2, XOA11, and XOA12 after 8 h in nonpermissive conditions. The inserts in each panel are digital magnifications of selected regions of the electron micrographs (indicated by the dotted-line squares) showing the accumulation of membranous material, the presence of empty cells, and cell division abnormalities in more detail. Bars, 0.5 μ m.

group of bacteria, which may explain at least in part their extreme resistance to APs. Recent structural studies of the lipid A moiety of *B. cepacia* (formerly genomovar I) and *B. mallei* revealed that only a proportion of lipid A species are nonstoichiometrically replaced with Ara4N (5, 31), although we do not know whether this is also the case for the lipid A from *B. cenocepacia*. Perhaps not all the lipid A molecules are required to be replaced with Ara4N to maintain the stability of the outer membrane. Furthermore, our results did not conclusively establish whether or not Ara4N is essential in the lipid A and/or the inner core oligosaccharide. The morphological defects of the mutants under nonpermissive conditions are strikingly similar to those found in *Escherichia coli* mutants with defects in the Imp/RlpB complex, which is essential for proper assembly of LPS in the outer membrane (33). Therefore, it is possible that the products encoded by the putative *arn* gene cluster in *B. cenocepacia* are also required for the assembly of LPS in this bacterium. A detailed biochemical analysis of the lipid A biosynthesis in *B. cenocepacia*, currently under way in our laboratories, will help to elucidate the role of these genes and will also confirm their predicted function in the synthesis of Ara4N.

We thank J. Bartholdson and J. Schmitt for technical assistance and M. Holden and J. Parkhill for giving us access to the preliminary annotation of the strain J2315. The electron microscopy was conducted in the Electron Microscopy Research Facility of the Schulich School of Medicine and Dentistry, University of Western Ontario, Canada, with the expert assistance of J. Sholdice.

A.R.B. is supported jointly by the Big Lottery Fund of the United Kingdom and the Cystic Fibrosis Trust. R.S.F. and S.A.L. are supported by graduate scholarships from the Canadian Cystic Fibrosis Foundation and Canadian Institutes of Health Research, respectively. This work was supported by grants from the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research (to M.A.V.). D.J.C. thanks the Royal Society of Edinburgh. M.A.V. holds

a Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.

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